REMARKS

Claims 12-19, 24-39, 43-47, 50-59, and 62-63 are pending in the application. Claims 12-19, 24-29, 40 and 43-45 have been withdrawn from further consideration. Claims 30-39, 46-47, and 50-59 have been examined on the merits. Support for the newly added claims 62 and 63 can be found at *inter alia*, page 12 in the Specification. Accordingly, no new matter has been inserted into the application. No new issue has been raised requiring further search or consideration.

Rejection Under 35 U.S.C. § 103(a) Over Hellmann in view of Moon, LaPlante, Hu '062 (U.S. Patent No. 6,107,062), and Gewirtz (Proc. Natl. Acad. Sci. 1996. v. 93, pp. 3161-3163)

Claims 22, 23, 30-39, 41, 42, and 46-61 have been rejected as being obvious over Hellmann in view of Moon, LaPlante, Hu '062 and Gewirtz. Applicants traverse this rejection. Reconsideration and withdrawal thereof are respectfully requested.

Hellmann

Hellmann discloses an M13 molecule with a Tobacco Vein Mottling Virus (TVMV) insert sequence. Hellmann further discloses performing DNA:RNA hydridization assays with the M13 molecule in a reticulocyte lysate cell-free translation system, the so called hybrid-arrested translation system.

Hellmann fails to disclose or suggest mixing the M13 molecule with a transfection effective composition containing lipids such as cationic lipids or liposomes because Hellmann's assays with the M13 molecule is conducted entirely in a cell-free system, and there is no disclosure or suggestion found in Hellmann to include any cell transfection reagent for introducing the M13 molecule into a eukaryotic cell.

Moon

Moon discloses a ribbon-type antisense oligonucleotide of about 120 nucleotides mixed with a transfection reagent.

LaPlante

LaPlante discloses transfecting a cell with cDNA that expresses mRNA encoding CHERP.

Hu '062

Hu '062 discloses a cell-based system for inhibiting target gene expression in which the cells are transfected with a double-stranded plasmid.

Gewirtz

Gewirtz discloses that lipid transfection agents can be mixed with oligodeoxynucleotides and plasmid DNA.

Distinctions of the present invention over the cited references

Basic considerations which apply to obviousness rejections

When applying 35 U.S.C. 103, the following tenets of patent law must be adhered to:

(A) The claimed invention must be considered as a whole;

(B) The references must be considered as a whole and must suggest the desirability and thus the obviousness of making the combination;

(C) The references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and

(D) Reasonable expectation of success is the standard with which obviousness is determined. *Hodosh v. Block Drug Co., Inc.*, 786 F.2d 1136, 1143 n.5, 229 USPQ 182, 187 n.5 (Fed. Cir. 1986). (MPEP 2141).

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant"s disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The initial burden is on the examiner to provide some suggestion of the desirability of doing what the inventor has done. "To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." *Ex parte Clapp*, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985). MPEP 2142.

Obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. "The test for an implicit showing is what the combined teachings, knowledge of one of ordinary skill in the art, and the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in

the art." *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000). See also *In re Lee*, 277 F.3d 1338, 1342-44, 61 USPQ2d 1430, 1433-34 (Fed. Cir. 2002) (discussing the importance of relying on objective evidence and making specific factual findings with respect to the motivation to combine references); *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988); *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992). MPEP 2143.

In order to establish *prima facie* obviousness of the invention over the cited references, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine reference teachings. The Federal Circuit has produced a number of decisions overturning obviousness rejections due to a lack of suggestion in the prior art of the desirability of combining references, as discussed in the aforementioned section. In the present situation, the Examiner has failed to establish *prima facie* obviousness of the present invention over Hellmann, Moon, LaPlante, Hu '062 and Gewirtz.

Hellmann's disclosure and the problem to be solved

Hellmann discloses an M13 construct with a Tobacco Vein Mottling Virus insert sequence, which is used to create DNA:RNA hybrid as used in an *in vitro* cell-free hybrid arrest assay. Hellmann is focused on developing an assay system to determine the origin of the polypeptide product encoded by the 5'- terminal region of the RNA of the potyvirus. Hellmann is further focused on precisely mapping the potyviral proteins and understanding the translational mechanisms by which they are produced. Hellmann states that single-stranded DNAs derived from plasmids containing approximately 95% of the sequences of TVMV RNA arrest the translation of specific portions of TVMV RNA and discovered that synthesis of P75 is initiated

near the 5' terminus (paragraph bridging pages 23-24). To solve the problem of understanding the translational mechanisms of potyvirus, Hellmann chose to employ a cell-free translation system using single-stranded DNA fragments obtained from M13, which contains the TVMV inserts because "initial attempts to perform hybrid-arrested translation experiments using double-stranded recombinant plasmids were unsuccessful due to rapid annealing of the DNA strands under the hybridization conditions use." (Page 25, paragraph bridging left and right columns).

Hellmann's research focus is on using a single-stranded DNA in cell-free hybrid arrested translation experiments because Hellmann is interested in the problem of solving the translational machinery of potyvirus for which the cell-free system is a more helpful experimental methodology than cell-based methodology. Accordingly, Hellmann fails to disclose or suggest transfecting any eukaryotic cell and therefore fails to disclose or suggest mixing its M13 molecule with a transfection agent.

Hellmann is not combinable with Moon, LaPlante, Hu '062, or Gewirtz

Applicants submit that the Hellmann reference and the Moon reference fail to be combinable with each other. The Moon reference discloses a composition that includes a 116-mer stem-loop DNA structure and a transfection effective carrier. Since Hellmann discloses only a cell-free system, with its own set of experimental challenges, there would be no reason for a person of ordinary skill in the art reviewing the Hellmann reference to consult the Moon reference directed to gene expression within a cell. Cell-free (Hellmann) and cell-based (Moon) systems each present separate, unique challenges. A person of skill in the art contemplating carrying out a cell-free based expression studies according to Hellmann would not look to a cell-

based gene expression system such as disclosed in Moon for guidance. Therefore, the Hellmann and Moon references are not analogous art and are not combinable.

Moreover, Moon states at page 4652, penultimate paragraph as follows:

> We used cationic liposomes to enhance the cellular uptake of RiAS oligos. From the experience of our own and other groups, a meaningful level of AS oligo uptake should be consistently obtainable when carried into cells by liposomes, regardless of the size of AS oligos (31, 32). Therefore, the relatively large size or RiAS oligos should not pose a problem for efficient cellular uptake. (emphasis added)

Moon suggests that the 116-mer oligonucleotide (RiAS) is relatively large, and that even this large an oligonucleotide should be able to be transfected into the cell. Moon considers 116-mer to be large. And yet makes no mention of the desirability or capability of transfecting a large circular single-stranded nucleic acid that is at least 3,000 bases long into a mammalian cell. Thus, it cannot be fairly said that the 116-mer transfection attained in Moon alone is suggestive of transfection effectiveness or desirability of a large circular single-stranded nucleic acid that is at least 3,000 bases long. Oligonucleotides, double stranded nucleic acids, linear nucleic acids, large circular single-stranded nucleic acids and so on are each biochemically, conformationally and sterically unique. There is a level of unpredictability as to how they would behave and whether they would be useful inside of a cell. Therefore, since Moon does not disclose or suggest transfection appropriate to the large circular single-stranded nucleic acid of the claimed invention, Moon fails to be applicable to the presently claimed invention.

LaPlante's disclosure of a human CHERP gene cDNA cloned into a plasmid and producing mRNA does not provide any motivation to transfect a large circular single-stranded nucleic acid molecule into a mammalian cell as in the claimed invention. LaPlante essentially discloses two types of nucleic acids – plasmid DNA and linear RNA. since LaPlante does not

disclose or suggest transfection appropriate to the large circular single-stranded nucleic acid of the claimed invention, LaPlante fails to be applicable to the presently claimed invention. Furthermore, Hellmann and LaPlante are again not combinable as references because they are in non-analogous art of cell-free and cell-based assay systems.

Applicants submit that the Hellmann reference and the Hu '062 patent fail to be combinable with each other. Hu '062 is firmly focused on inhibiting target gene expression by the expression of exogenously introduced plasmid DNA that expresses antisense RNA. Hu '062's research field is limited to the realm of transfecting cells, assaying for gene expression within a cell background, and assaying for changes in cell morphology. The methods and techniques employed richly revolve around cell cultures and assays using live organisms, which extend to therapeutics and treatment of disease, specifically AIDS. This is in stark contrast to the Hellmann reference, which is directed to a cell-free assay system that employs a single-stranded M13 phage construct to determine the translational mechanism of the potyvirus. Hellmann fails to disclose any information regarding any cell-based type of system. And a person in the art of target gene inhibition by expression of antisense RNA would not look to a cell-free assay system for guidance in solving its problems.

Since the purposes for which each reference uses either the single-stranded or double-stranded form of either the phage or the plasmid vector are divergent, a person of ordinary skill in the art reviewing the Hellmann reference would not be motivated to consider using a plasmid DNA expressing antisense RNA to assist in solving the hybridization problem discussed in the Hellmann reference. And *vice versa*, a person in the cell-based antisense therapy field would not be motivated to consider using a single-stranded M13 vector construct of Hellmann in solving its

therapeutic focus, as there is simply no motivation found in either reference to combine these references.

The Gewirtz reference discloses transfecting oligonucleotides and double stranded plasmid DNA into mammalian cells by complexing these types of nucleic acids with a transfection effective carrier. Gewirtz is concerned with better efficiency of oligonucleotides because this was the major focus of antisense research at the time. Transfection effective agents for nucleic acids were known in the art at the time of the invention as exemplified by Gewirtz. It is noted however that Gewirtz makes no mention expressly or impliedly that a large circular single-stranded nucleic acid as in the claimed invention may be transfected into eucaryotic cells. Furthermore, again as with the other cited references, the Gewirtz reference discloses a cell-based transfection oriented technology, which is not analogous to the cell-free system that Hellmann discloses. Therefore, these references fail to be combinable with each other.

Hindsight reconstruction

Applicants submit that the Examiner has cobbled together the cited five (5) references in an attempt to show obviousness of the claimed composition. The Examiner has cited these references with hindsight vision afforded by the claimed invention. Clearly, in order to establish obviousness of the claimed composition, it is not enough to show that each of the separate ingredients are in existence, as the Examiner has done. The Examiner must provide references that show the desirability and the motivation for combining the references to arrive at the claimed composition. All of the cited references fall short of this simply because none of the cited references recognizes or appreciates that the effective usefulness of transfecting these large circular single-stranded nucleic acid molecules into eucaryotic cells.

Indeed, Hellmann shows the opposite of the inventive concept because Hellmann shows a large circular single stranded nucleic acid molecule that is used in a cell-free system. Therefore, Hellmann certainly fails to provide any motivation to insert its nucleic acid into a eukaryotic cell, and further, Hellmann is not applicable to the cell-based transfection system of the claimed invention.

It must be appreciated that Applicants have at the time of the invention demonstrated for the first time that transfecting these large circular single-stranded nucleic acids into eukaryotic cells resulted in useful and effective antisense effects, which was not recognized before in the art because the art at the time was focused on injecting small oligonucleotides. No one had appreciated that a large circular single-stranded nucleic acid such as instantly claimed could be used to effect an antisense response.

There is no motivation found in the cited references to make the claimed composition

The Examiner's comments in the Office Action of July 13, 2005 have been noted. To summarize, the Examiner asserts that because it is known in the art that a closed circular approximately 120 nucleotide sequence can be transfected into eucaryotic cells (Moon); a plasmid DNA can be transfected into eucaryotic cells (LaPlante); a plasmid that expresses several target-specific antisense RNA can be transfected into eucaryotic cells (Hu); and generally, oligodeoxynucleotides and plasmid DNA can be mixed with lipid transfection agents (Gewirtz), it would have been obvious to mix the large circular single-stranded molecule disclosed in Hellmann with a transfection reagent at the time of the invention. In addition, the Examiner believes that a person of ordinary skill in the antisense art would understand that the transfection effective agents are commonly used with antisense nucleic acids. In essence, the Examiner

believes that all nucleic acids have similar properties, and therefore, if a eucaryotic transfection agent can be mixed with some of these types of nucleic acids, then the transfection agents can be mixed with the large circular single-stranded nucleic acid of the present invention as well.

Applicants disagree with the Examiner's analysis of the implications of the cited prior art and the reasons for the rejection. Applicants were the first to show that mixing the inventive large circular antisense nucleic acid molecule with a transfection effective reagent and transfecting the eucaryotic cell has the effect of ablating gene expression of the corresponding gene in the cell. Prior to Applicants' demonstration of this effect, there was no suggestion in the prior art that a large circular single-stranded nucleic acid as in the claimed invention was desirable for transfection into a eucaryotic cell to achieve any purpose at all. While double-stranded plasmids and oligonucleotide nucleic acids were known to be transfected into eucaryotic cells to achieve either therapeutic or other gene expression purposes, there was no motivation to insert a large circular single-stranded nucleic acid as in the claimed invention into eucaryotic cells.

The Examiner lumps all of the antisense molecules into one group and concludes that if some of these antisense molecules are known to be transfected into eucaryotic cells, then it would be obvious to mix the large circular antisense molecule of the present invention with a transfection agent for carrying out transfection into eucaryotic cells. However, Applicants note that not all antisense molecules are the same nor should they be lumped together into a single group. An oligonucleotide has unique properties, as does a double-stranded plasmid. In this regard, there has been no instance in the prior art that indicates a desirability of transfecting a single-stranded version of the large circular nucleic acid as in the claimed invention. Turning to

the Gewirtz reference for the moment, Applicants point to page 3161, far left column, last three sentences of the first paragraph, in which it is stated:

> A larger body of work has focused on the anti-mRNA or so-called "anti-sense" strategies, composed principally of the use of ribozymes and antisense oligodeoxynucleotides (AS ODNs). Antisense oligonucleotides have received the majority of attention because of their apparent ease of synthesis and use.

As can be seen in this passage, essentially there were two types of antisense strategies at the time of the invention—using the enzyme ribozyme, and using antisense oligonucleotides. The oligonucleotides were used mainly because of ease of synthesis. Because of the focus on the antisense oligonucleotides, a person of skill in the art of antisense therapy or diagnostics would be directed to antisense oligonucleotides and not to large circular single-stranded nucleic acid molecules as in the presently claimed invention for insertion into eucaryotic cells. Therefore, there is no reason at the time of the invention to mix a transfection reagent with the large circular single-stranded nucleic acid molecule of the invention because there is no desire, intent, or goal of inserting the inventive molecule into a eucaryotic cell. This is a significant point. If there is no motivation or directed reason to combine the particular type of compound of the instant claims with the transfection agent, then the composition as claimed cannot be held to be "obvious". This is so, especially in view of the state of the art at the time of the invention when it was thought that small oligonucleotides were most desirable for insertion into the eucaryotic cells. Thus, at a minimum, there was no motivation to insert the inventive compound into a eucaryotic cell. And further, it would be reasonable to conclude that a reference such as Gewirtz steers toward optimizing small oligonucleotide transfection, and therefore teaches away from using the inventive composition.

Applicants note that the large circular single-stranded nucleic acid of the invention cannot be grouped with the general antisense molecule that the Examiner appears to have categorized. Clearly, a double-stranded molecule has different properties from a single-stranded molecule. Even though they may possess nucleotide bases as the common unit components, the biochemical characterization of a single-stranded and double-stranded molecule reveal different results. Further, oligonucleotides in the range of 20 to 150, for instance, are not comparable to a large molecule that has over 3,000 bases. The molecular dynamics between these types of molecules are different. Just based on the sheer difference in size, a person of skill in the art would expect them to behave differently.

Applicants submit for the Examiner's consideration in support of the patentability of the presently claimed invention, a recently published article in *Nature Biotechnology* (Lee et al. "Gene knockdown by large circular antisense for high-throughput functional genomics", *Nature Biotechnology*, Vol. 23, No. 5, May 2005) by the inventors regarding the subject matter of the present application. *Nature Biotechnology* is one of the most prestigious journals in the field of biotechnology. Therefore, the subject matter of the claimed invention is acknowledged in scientific circles to be of significant advance over existing knowledge surrounding the antisense technology, fully supporting the non-obviousness of the presently claimed invention. Accordingly, the presently claimed invention is patentable over the cited references.

It is believed that the application is now in condition for allowance. Applicants request the Examiner to issue a notice of Allowance in due course. The Examiner is encouraged to contact the undersigned to further the prosecution of the present invention.

The Commissioner is authorized to charge JHK Law's Deposit Account No. **502486** for any fees required under 37 CFR §§ 1.16 and 1.17 that are not covered, in whole or in part, by a

credit card payment enclosed herewith and to credit any overpayment to said Deposit Account No. **502486**.

Respectfully submitted,

JHK Law

Dated: October 13, 2005

By: _____
Joseph Hyosuk Kim, Ph.D.
Reg. No. 41,425

P.O. Box 1078
La Canada, CA 91012-1078
(818)249-8177 – direct
(818)249-8277 – fax

Enclosure: Lee et al. "Gene knockdown by large circular antisense for high-throughput functional genomics", *Nature Biotechnology,* Vol. 23, No. 5, May 2005

nature biotechnology

Gene knockdown by large circular antisense for high-throughput functional genomics

Yun-Han Lee[1,4,6], Ik-Jae Moon[1,6], Bin Hur[1,6], Jeong-Hoh Park[1], Kil-Hwan Han[1], Seok-Yong Uhm[1], Yong-Joo Kim[1], Koo-Jeong Kang[2], Jong-Wook Park[3], Young-Bae Seu[4], Young-Ho Kim[4] & Jong-Gu Park[1,5]

Single-stranded genomic DNA of recombinant M13 phages was tested as an antisense molecule and examined for its usefulness in high-throughput functional genomics. cDNA fragments of various genes (*TNF-α*, *c-myc*, *c-myb*, *cdk2* and *cdk4*) were independently cloned into phagemid vectors. Using the life cycle of M13 bacteriophages, large circular (LC)-molecules, antisense to their respective genes, were prepared from the culture supernatant of bacterial transformants. LC-antisense molecules exhibited enhanced stability, target specificity and no need for target-site searches. High-throughput functional genomics was then attempted with an LC-antisense library, which was generated by using a phagemid vector that incorporated a unidirectional subtracted cDNA library derived from liver cancer tissue. We identified 56 genes involved in the growth of these cells. These results indicate that an antisense sequence as a part of single-stranded LC-genomic DNA of recombinant M13 phages exhibits effective antisense activity, and may have potential for high-throughput functional genomics.

Gene expression can be specifically reduced or ablated in cells after the uptake of antisense molecules complementary to a specific mRNA sequence. Antisense inhibition of gene expression is believed to be achieved through RNaseH activity after the formation of an antisense DNA-mRNA duplex or through steric hindrance of movement/binding of the ribosomal complex[1]. Gene silencing by antisense treatment has been considered ideal for functional analysis of genes and further for drug target discovery[2]. Intense efforts have been made to develop antisense anticancer agents that eliminate aberrant expression of genes involved in tumor initiation and progression[3–8]. The efficacy of antisense oligonucleotides (AS-oligos) has been validated in animal models[9–14].

We have previously described a series of distinct antisense molecules, with closed structures lacking exonuclease active sites, resulting in much enhanced stability in biologic fluids[15,16]. These results prompted us to investigate the potential of the single-stranded circular genome of M13 bacteriophages (phages) as antisense molecules. A recombinant M13 phagemid vector was engineered to produce a single-stranded circular genome containing an antisense sequence, which was then tested for enhanced stability and specific antisense activity.

Various methods have been devised to study gene expression[17–23], however, the information generated has been limited to differential or sequential expression profiles of genes in different tissues or cells. Rapid accumulation of genomic sequence information and expression profiling has created a bottleneck in subsequent definitive gene functionalization and/or target validation. Most definitive functionalization of genes has been performed with various conventional gain-of-function or loss-of-function studies. Loss-of-function studies have been done either with gene knockdown using conventional antisense[24,25] or its related technologies[26–28], or with gene knockout using homologous recombination[29,30]. These approaches are limited in that they must be done individually. Construction of an extensive antisense library may provide an answer to this information bottleneck for massive gene functionalization. AS-oligo libraries have been partially established and used to obtain functional data of a large number of genes. Constructing such a library, however, can be costly and time consuming because a target site search must be done[31–33]. Thus, another approach is needed to facilitate construction of an antisense library. LC-antisense constructs may provide a salient advantage in library construction because they do not require target site searches.

In the present study, we tested the efficacy of single-stranded circular genomic DNA of M13 phagemids as antisense molecules with regard to enhanced stability and target-specific reduction of gene expression. An LC-antisense library was constructed by using cDNA prepared from hepatoblastoma tissue by subtractive hybridization, which was then used to screen genes involved in the growth of a liver cancer cell line using a high-throughput approach.

RESULTS

Construction and purification of LC-antisense molecule

Covalently closed circular antisense molecules are unusually stable and effective in reducing target gene expression, suggesting the potential of the single-stranded circular genome of bacteriophage M13 as an antisense molecule. The F1 replication origin of the M13 phagemid

[1]WeIGENE Inc., 71B 4L, Development Sector 2-3, Sungseo Industrial Park, Dalseogu, Daegu, 704-230, South Korea. [2]Department of General Surgery, Dongsan Medical Center, Keimyung University, [3]Department of Immunology, Keimyung University School of Medicine, [4]Department of Microbiology, College of Natural Sciences, Kyungpook National University, 1370 Sangyeokdong, Bookgu, Daegu, 702-701, South Korea. [5]Department of Medical Genetic Engineering, Keimyung University School of Medicine, Dongsan Medical Center, 194 Dongsandong, Joonggu, Daegu, 700-712, South Korea. [6]These authors contributed equally to this work. Correspondence should be addressed to J.-G.P. (jonggu@kmu.ac.kr).

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was used to generate a single-stranded circular phage genome harboring either the antisense or sense sequence for a target gene. Rat *TNF-α* cDNA was cloned into a pBS KS (−) vector to produce the antisense sequence as a part of the phage genome (see **Supplementary Fig. 1** online). The phage genomic LC-antisense molecule, designated as *TNFα*-LCAS in this study, was isolated from the culture supernatant of bacterial cells that were transformed with recombinant phagemid harboring rat *TNF-α* cDNA[34]. Large-scale purification of the LC-antisense molecule was done by gel filtration column chromatography. The antisense sequence in the single-stranded phage genomic DNA was confirmed by DNA sequencing using the T3 primer (data not shown). LC-antisense molecules to the *c-myc*, *c-myb*, *cdk2* and *cdk4* genes were also constructed by the same approach and designated as *c-myc*-LCAS, *c-myb*-LCAS, *cdk2*-LCAS and *cdk4*-LCAS, respectively. Similarly, both LCSE (single-stranded phage genome containing the sense sequence of each target gene) and LCSS (single-stranded phage genome devoid of an insert sequence) were also prepared as control molecules.

LC-antisense molecules were expected to be resistant to exonucleases because of their circular structure[15,16]. When single-stranded (ss) *TNFα*-LCAS was incubated with either *Xho*I or exonuclease III, the antisense molecules were found to be largely intact after 3 h (**Fig. 1a**). In contrast, when *Xho*I was added to the double-stranded (ds) recombinant M13 phagemid DNA harboring the *TNF-α* cDNA, the dsDNA was restriction digested, generating two linear bands of 3.4 and 0.5 kb on an agarose gel. Furthermore, the dsDNA was digested to completion by the combination of *Xho*I and exonuclease III, leaving no detectable DNA band. The fact that *TNFα*-LCAS is ssDNA was reconfirmed by the efficient digestion of the circular molecules with S1 nuclease, which specifically cuts ssDNA regardless of sequence composition. When *TNFα*-LCAS was combined with cationic lipids, a large fraction of the antisense molecules remained intact after an extended period of incubation in fetal bovine serum (FBS), even after 24 h incubation in 30% FBS (**Fig. 1b**).

Effective inhibition of target gene expression by LC-antisense

Encouraged by the enhanced tolerance of LC-antisense molecules to nucleases, we tested *TNFα*-LCAS for antisense activity. *TNFα*-LCAS (1.4 nM) was complexed with cationic lipids and added to the rat monocytic WRT7/P2 cell line in which *TNF-α* expression was induced by lipopolysaccharide (LPS) treatment. When treated with *TNFα*-LCAS, the cells were shown to have a substantially reduced level of *TNF-α* mRNA (**Fig. 1c**). In contrast, cells treated with either *TNFα*-LCSE (the sense strand of *TNF-α* DNA) or LCSS (single-stranded vector genomic DNA) did not show much reduction of *TNF-α* mRNA. The RT-PCR band of *TNF-α* was confirmed by Southern hybridization with a probe that bound to the internal region of the amplified DNA fragments (**Fig. 1c**). To confirm that the treatment of LC-antisense leads to the eventual blockade of protein synthesis from target mRNA, we transfected WRT7/P2 cells with *TNFα*-LCAS and measured the level of TNF-α protein secreted from the transfectants. Commensurate with the reduction of *TNF-α* mRNA level, the level of TNF-α in the cell culture supernatant was also reduced by more than 90% after treatment with *TNFα*-LCAS (**Fig. 1d**). In contrast, none of the two control molecules, *TNFα*-LCSE and LCSS, significantly reduced the level of TNF-α protein in WRT7/P2 transfectants. After observing the effective antisense activity of *TNFα*-LCAS, we performed experiments to determine if LC-antisense molecules to other genes, such as *c-myc* and *c-myb*, would also block expression of their respective target genes. When 1.12 nM of *c-myc*-LCAS was added to K562 cells, *c-myc* mRNA was reduced by about 70% compared to that obtained after *c-myc*-LCSE transfection (**Fig. 1e**). Similarly, treatment of 1.12 nM of *c-myb*-LCAS to K562 cells reduced *c-myb* mRNA level by about 80% (**Fig. 1f**). The treatment of *c-myc*-LCAS did not affect the expression of the *c-myb* gene and vice versa (**Fig. 1e,f**). These results show that LC-antisense can efficiently reduce gene expression in smaller amounts than most conventional antisense molecules.

Target specificity and antisense activity of LC-antisense

If LC-antisense is to be effective, it must be target specific, especially in regard to its large length. Thus, sequence specificity of LC-antisense molecules was examined by an RNase protection assay (RPA) after treatment of HeLa cells with *cdk2*-LCAS. Whereas *cdk2*-LCAS reduced *cdk2* expression in the cells at time points of 24 and 48 h, the antisense did not substantially affect expression of other genes, *cdk1*, *p16*, *L32* and *GAPDH* (**Fig. 2a**). CDK2 levels in HeLa cells transfected with *cdk2*-LCAS were also examined by western blotting analysis. Whereas *cdk2*-LCAS at a concentration of 0.8 or 1.6 nM reduced the intracellular level of CDK2 by more than 80%, an equal amount of *cdk2*-LCSE

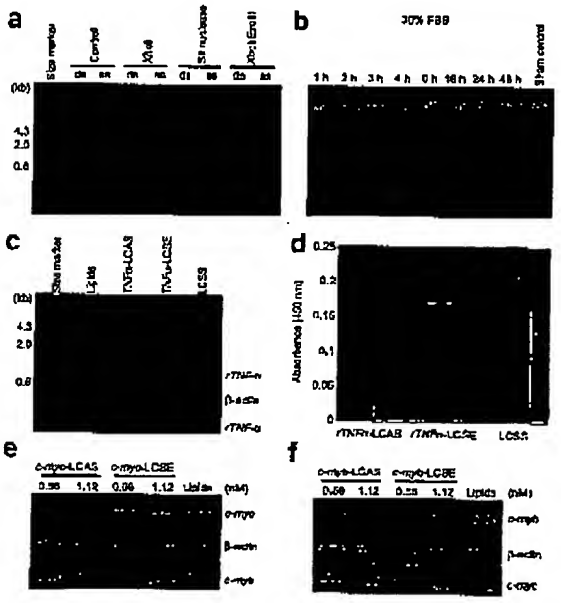

Figure 1 Stability and antisense activity of LC-antisense. (a) Characterization of *TNFα*-LCAS molecules. Either double-stranded (ds) recombinant *TNFα*-phagemid or single-stranded (ss) *TNFα*-LCAS was incubated with the restriction endonuclease *Xho*I, S1 nuclease or *Xho*I/exonuclease III and run on a 1% agarose gel with sham-treated controls. (b) Stability test of LC-antisense molecules. *TNFα*-LCAS plus cationic lipid complexes were treated with 30% FBS for different periods of time as indicated and run on a 1% agarose gel with a sham-treated control. (c) Antisense activity of *TNFα*-LCAS on *TNF-α* mRNA levels in WRT7/P2 cells. RT-PCR analysis was carried out with two sets of primers, either *TNF-α* primers or β-*actin* primers. Southern blotting, shown in the bottom panel, was carried out to detect *TNF-α* expression. (d) Reduced expression of TNF-α by *TNFα*-LCAS treatment. ELISA of TNF-α in medium: WRT7/P2 cells transfected with *TNFα*-LCAS, *TNFα*-LCSE or LCSS. Each bar value represents the mean ± s.d. of triplicate experiments. Statistical significance was calculated with student's *t*-test (analysis of variance, * $P < 0.05$). (e,f) Indicated amounts of LC-antisense molecules to *c-myc* (e) and *c-myb* (f) were transfected into K562 cells. Amplified PCR fragments of each target gene were run on a 1% agarose gel and visualized with ethidium bromide staining.

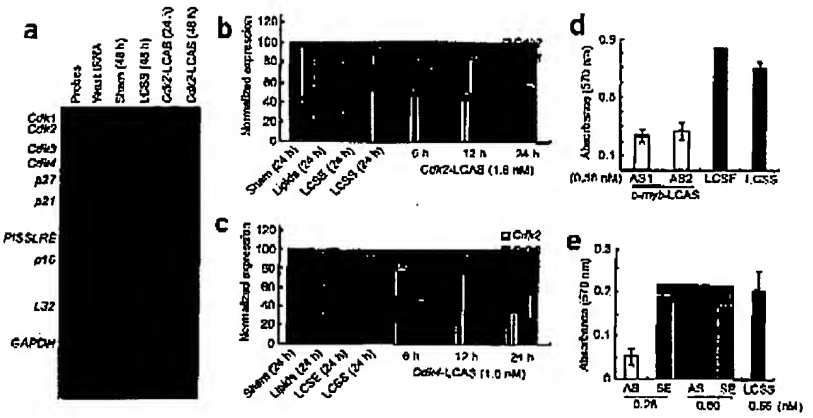

Figure 2 Target specificity and antisense activity of LC-antisense. Detection of gene expression was done after the transfection of LC-antisense into HeLa. (a) RPA assay for expression of various genes in HeLa cells. Total RNA was extracted at 24 h or 48 h after treatment with *cdk2*-LCAS or at 48 h after sham treatment or LCSS treatment in 6-well plates. After hybridization of the extracted RNA with biotin-labeled probes or yeast tRNA (negative control), the samples were run together with unhybridized probes as markers on a denaturing polyacrylamide gel. Reference mRNAs: *L32* and *GAPDH*. Irregular splotches are shown on *L32* and *GAPDH* bands of the two right-hand lanes. PISSLRE, a human CDC-2 related protein kinase. (b,c) Real-time RT-PCR analysis of *cdk* gene expression in HeLa cells treated with *cdk2*-LCAS (b) or *cdk4*-LCAS (c). Total RNA was extracted at 6 h, 12 h and 24 h after treatment with 1.6 nM of LC-antisense or at 24 h after sham, lipids alone, LCSE and LCSS treatments in 24-well plates. In all real-time RT-PCR experiments, expression is calculated relative to β-actin and is normalized to sham treatment. Each bar value represents the mean ± s.d. of triplicate experiments. (d,e) Effect of LC-antisense on proliferation of human cancer cell lines was measured by MTT assays after transfection of LC-antisense molecules. (d) Effects of two types of *c-myb*-LCAS on K562 cell proliferation. *C-myb*-LCAS1 and *c-myb*-LCAS2 contain 0.5 kb or 1.5 kb of the *c-myb* cDNA sequence, respectively. (e) *Cdk4*-LCAS on MCF-7 cell proliferation. *AS*, *cdk4*-LCAS; *SE*, *cdk4*-LCSE. Each bar value represents the mean ± s.d. of triplicate experiments.

had no substantial effect (see **Supplementary Fig. 2** online). Interestingly, the level of *cdk4* mRNA was also reduced by the treatment of *cdk2*-LCAS. The result may be explained by the coordinate regulation between the two G1 phase-specific cell cycle regulators in a sequential fashion[35,36]. However, it was necessary to rule out the off-target effect of *cdk2*-LCAS, therefore, we monitored the changes in *cdk2*, *cdk4* and *cdk6* gene expression after either *cdk2*- or *cdk4*-LCAS treatment at earlier time points using real-time quantitative RT-PCR. *Cdk4* shares conserved regions in some of its sequence to those of *cdk2* and *cdk6*, the other two cell cycle regulators. Nucleotides 749–848 of the *cdk4* cDNA (GenBank accession number NM_000075) exhibits 75% sequence similarity to a region of the *cdk2* gene (NM_001798) and 74% to a region of *cdk6* gene (NM_001259), respectively. There is, however, no sequence similarity between *cdk2* and *cdk6* genes. A time-course experiment was initially carried out by transfection of 2×10^4 HeLa cells by various amounts of *cdk2*- or *cdk4*-LCAS to monitor the optimal concentration of the antisense and time points for effective knockdown of their respective gene expression. In addition, the activity of *cdk2*-LCAS was compared to that obtained with *cdk2* siRNA. When 0.8, 1.6 and 2.4 nM of either *cdk2*- or *cdk4*-LCAS was added to HeLa cells, target mRNA was reduced by about 40–75%, in a dose-dependent manner at 6, 12 and 24 h (see **Supplementary Fig. 3a,b**). In contrast, cells that were treated with either LCSE or LCSS did not show substantial reduction of either *cdk2* or *cdk4* gene expression. The influence on the expression of noncognate *cdk* genes was then examined after *cdk2*- and *cdk4*-LCAS treatment, respectively. When 1.6 nM of *cdk2*-LCAS was added to HeLa cells, the level of *cdk4* mRNA was not changed substantially at 6 h and 12 h, but was decreased by about 48% at 24 h (**Fig. 2b**). Similarly, 50 nM *cdk2* siRNA reduced target *cdk2* RNA by about 48% compared to the sham treatment, but *cdk4* RNA was not reduced at earlier time points. However, after the 24 h incubation with *cdk2* siRNA, *cdk4* mRNA was reduced by approximately 47% (see **Supplementary Fig. 3c** online).

Further, when 1.6 nM of *cdk4*-LCAS was added to HeLa cells, the expression of *cdk2* and *cdk6* mRNA was not affected at 6 h and 12 h, but was decreased by approximately 64% and 47% at 24h, respectively (**Fig. 2c**). The lack of off-target expression interference among the functionally associated genes, *cdk2*, *cdk4* and *cdk6*, at earlier time

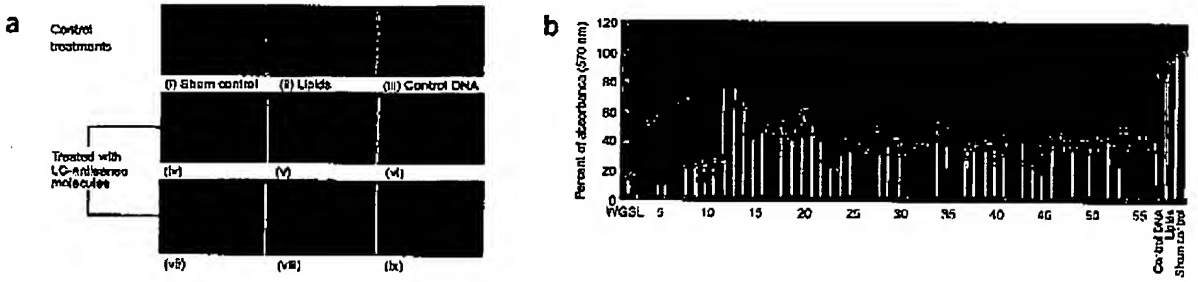

Figure 3 High-throughput functional analysis to identify genes involved in the growth of liver cancer cells. (a) Growth inhibition of HepG2 cells after transfection with LC-antisense library was examined by light microscopy 4 d after transfection (200× magnification). (i)–(iii), control treatments as indicated; (iv)–(ix), HepG2 cells treated with different LC-antisense molecules. A representative example of the data acquired from treatments with 6 out of 1,200 kinds of LC-antisense is shown. (b) LC-antisense species of 56 random genes were transfected to a HepG2 cell line in a macroarray configuration. The transfectants were examined for growth inhibition by MTT assays in triplicate. Cells that were sham-treated, treated with lipids alone and treated with control DNA plus lipid complexes were assayed simultaneously. Each bar value represents the mean ± s.d. of triplicate experiments.

Table 1 List of genes involved in liver cancer cell growth

Gene description and putative functional category	No. of clones	Accession no.
Protein synthesis		
Homo sapiens ribosomal protein S25 (RPS25)	WGSL5	BC004986
Siboglinum ekmani 18S ribosomal RNA, partial sequence	WGSL16	AF315062
H. sapiens ribosomal protein S8 (RPS8)	WGSL19	NM_001012
H. sapiens ribosomal protein, large P1	WGSL23	NM_001003
H. sapiens ribosomal protein S24 (RPS24)	WGSL33	NM_033022
H. sapiens ribosomal protein S17 (RPS17)	WGSL38	M13932
H. sapiens clone IMAGE:3543815	WGSL39	BC020169
H. sapiens ribosomal protein L27 (RPL27)	WGSL45	NM_000988
H. sapiens ribosomal protein S5 (RPS5)	WGSL46	BC018151
H. sapiens ribosomal protein L35 (RPL35)	WGSL55	NM_007209
Translation factors		
H. sapiens eukaryotic translation initiation factor 3, subunit 6 interacting protein (EIF3S6IP)	WGSL3	NM_016091
H. sapiens eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2)	WGSL28	NM_001967
H. sapiens eukaryotic translation elongation factor 1 gamma	WGSL41	BC028179
Structural proteins and their regulators		
H. sapiens tissue inhibitor of metalloproteinase 1 (TIMP1)	WGSL6	XM_033878
H. sapiens clone MGC:5318 IMAGE:2900273	WGSL14	BC006781
H. sapiens beta-2-microglobulin (B2M)	WGSL32	NM_004048
H. sapiens syntaxin 7 (STX7)	WGSL53	XM_004526
Metabolism		
H. sapiens glutamate dehydrogenase 1 (GLUD1)	WGSL18	NM_005271
Human liver glutamate dehydrogenase	WGSL25	J03248
H. sapiens similar to serine (or cysteine) proteinase inhibitor	WGSL34	BC011991
Human mRNA for glutamate dehydrogenase	WGSL42	X07769
Other		
H. sapiens alpha-fetoprotein (AFP)	WGSL8	BC027881
H. sapiens ferritin, light polypeptide (FTL)	WGSL9	NM_000146
H. sapiens cutaneous T-cell lymphoma-associated tumor antigen se20-4 (SE20-4)	WGSL11	BC024270
H. sapiens apolipoprotein A-II	WGSL12	BC005282
H. sapiens heat shock 70 kDa protein 8 (HSPA8)	WGSL13	NM_006597

Table 1 continued on following page

points and between the *c-myb* and *c-myc* genes by respective LC-antisense molecules demonstrate the target specificity.

We next tested LC-antisense molecules for growth inhibition of cancer cell lines by targeting *c-myb* and *cdk4*. To accomplish this, two different LC-antisense molecules to *c-myb* at 0.56 nM (*c-myb*-LCAS1 containing 0.5 kb *c-myb* antisense and *c-myb*-LCAS2 containing 1.5 kb *c-myb* antisense) were added to K562. When the cancer cell lines transfected with the antisense molecules were examined for growth with the MTT assay, both *c-myb*-LCAS1 and *c-myb*-LCAS2 were able to inhibit cancer cell growth by more than 60% (Fig. 2d). In contrast, *c-myb*-LCSE and LCSS did not substantially affect K562 cell growth. Similarly, when *cdk4*-LCAS was added to MCF-7 cells, the cell growth of antisense transfectants was inhibited by more than 70% at antisense concentrations of either 0.28 or 0.56 nM. In contrast, *cdk4*-LCSE showed only marginal inhibition of cell growth, less than 9% and 15% inhibition at the same concentrations (Fig. 2e). The changes of CDK4 levels in MCF-7 cells treated with various amounts of *cdk4*-LCAS were also examined with western blot analysis. Whereas *cdk4*-LCAS at a concentration of 0.8 or 1.6 nM reduced the CDK4 level by more than 60%, an equal amount of *cdk4*-LCSE had no substantial effect (see Supplementary Fig. 4 online). These results demonstrate that LC-antisense molecules may provide target specificity and effective antisense activity.

En masse identification of liver cancer-related genes

The fact that a phagemid vector can be easily used to construct a cDNA library, prompted us to investigate the feasibility of high-throughput functional genomics using LC-antisense technology. Using an LC-antisense library can be appealing because target site searches are not required. Thus, we constructed an LC-antisense library to identify genes that are functionally involved in the growth of liver cancer cells (see Supplementary Fig. 5 online). To improve our chances of finding genes of interest, we prepared mRNA from both hepatoblastoma and noncancerous adjacent liver tissues, which was differentially amplified for liver cancer–specific mRNA by a suppression subtractive hybridization method[37]. Differentially amplified cDNAs were unidirectionally cloned into a phagemid vector, and the cDNA library constructed was transformed into *Escherichia coli* competent cells. From the cDNA library of 9,600 transformants, 1,200 clones with cDNA inserts of more than 500 base pairs were selected by a simplified method for plasmid isolation[38]. LC-antisense molecules were then purified from the culture supernatant of bacterial competent cells superinfected with helper bacteriophages. The random gene LC-antisense library of 1,200 member species was arrayed for transfection in 13 96-well plates that had been seeded with HepG2 cells for functional analysis. Each LC-antisense molecule (0.1 μg) was complexed with cationic lipids at a ratio of 1:3 (wt/wt) and transfected into 7×10^3 HepG2 cells in each well of 96-well plates. Cells were inspected for morphological changes with light microscopy (Fig. 3a) and measured quantitatively for growth inhibition with an MTT assay 4 d after transfection. Of the 1,200 antisense species selected by insert sizes, 153 (~13%) were found to be inhibitory to cancer cell growth in varying degrees. In contrast, cells treated with single-stranded control DNA (devoid of antisense insert sequences) exhibited a mild

Table 1 Continued

Gene description and putative functional category	No. of clones	Accession no.
H. sapiens haplotype M*2 mitochondrion	WGSL21	AF382013
H. sapiens haptoglobin (HP)	WGSL26	NM_005143
Human cytochrome P450IIE1 (ethanol-inducible) gene	WGSL30	J02843
H. sapiens cytochrome b5 outer mitochondrial membrane precursor	WGSL31	BC014431
Human DNA sequence from clone RP4-792D7 on chromosome 1q42.2-43. Contains the 5′ end of the TARBP1 gene for TAR (HIV) RNA-binding protein 1	WGSL35	AL136124
H. sapiens interferon, gamma-inducible protein 30 (IFI30)	WGSL36	XM_038146
H. sapiens fibrinogen, gamma polypeptide (FGG), transcript variant gamma-A	WGSL40	NM_000509
H. sapiens hypothetical protein MyD14 (MYD14)	WGSL44	NM_030918
Human liver fatty acid binding protein (FABP)	WGSL49	M10050
H. sapiens clone MGC:12445 IMAGE:3935036	WGSL50	BC005348
Human gene for heterogeneous nuclear ribonucleoprotein (hnRNP) core protein A1	WGSL51	X12671
H. sapiens FK506 binding protein 3 (25kD) (FKBP3)	WGSL52	NM_002013
Undefined functions		
Human chromosome 14 DNA sequence BAC R-123M6 of library RPCI-11 from chromosome 14	WGSL1	AL117190
Human DNA sequence from clone CTA-175E3 on chromosome 22q12.1	WGSL2	Z95113
Human DNA sequence from clone RP11-38P6 on chromosome 9	WGSL4	AL354874
H. sapiens BAC clone RP11-620E11 from chromosome 4	WGSL7	AC079926
H. sapiens hypothetical protein FLJ14075 (FLJ14075)	WGSL10	NM_024894
H. sapiens PRO2675 mRNA	WGSL15	AF119890
H. sapiens mRNA; cDNA DKFZp7628195	WGSL17	AL359585
H. sapiens clone RP11-56D18 from chromosome 2	WGSL20	AC019159
H. sapiens cDNA FLJ35730 fis, highly similar to alpha-1-antichymotrypsin precursor	WGSL22	AK093049
H. sapiens BAC clone RP11-449G13 from chromosome 16	WGSL24	AC020716
H. sapiens chromosome 5 clone RP11-412P18	WGSL27	AC091952
H. sapiens clone IMAGE:3923943	WGSL29	BC024924
H. sapiens chromosome 4 clone B366D24 map 4q25	WGSL37	AC004067
H. sapiens BAC clone RP11-360H4 from chromosome 2	WGSL43	AC019086
Human DNA sequence from clone RP11-334A14 on chromosome 1	WGSL47	AL445183
H. sapiens genomic MHC class III complement gene cluster (MCGC@) on chromosome 6	WGSL48	NG_000013
H. sapiens BAC clone CTD-2324K8 from 7p14-p13	WGSL54	AC011230
H. sapiens genomic DNA, chromosome 11q clone:RP11-660L20	WGSL56	AP001102

level of growth inhibition that could also be seen in cells treated with a dsDNA-lipid complex. To eliminate redundancy, we compared the sequences of cDNA clones complementary to the 153 growth-inhibiting LC-antisense molecules and searched the GenBank database for matching sequences. For instance, LCAS 3, 5, 8, 9, 10 and 23 contained the antisense sequences reversely complementary to the cDNA sequences of WGSL3 (nucleotides 1367–1885 of GenBank accession number NM_016091), WGSL5 (nt 21–489 of BC004986), WGSL8 (nt 1448–2017 of BC027881), WGSL9 (nt 421–863 of NM_000146), WGSL10 (nt 2119–2446 of NM_024894) and WGSL23 (nt 321–512 of NM_001003) genes, respectively. There were 56 unique sequences out of 153 cDNAs, and these were sorted and designated as clones WGSL 1–56. Putative functional categorization of each gene was then performed by motif-based searches on the basis of the revealed sequence information (Table 1). The LC-antisense molecules derived from the 56 clones were then designated as LCAS 1–56. Functional categorization indicated that 18 out of the 56 genes encode proteins of undefined functions. The remaining 38 genes have previously defined functions. The growth inhibiting activities of the 56 LC-antisense molecules were further confirmed by repetitive MTT assays (Fig. 3b). These 56 genes appear to have functions directly or indirectly related to cell growth of hepatoblastomas.

Effects of LC-antisense on cell cycle progression and apoptosis

The 56 LC-antisense species inhibitory to cancer cell growth were studied further to reconfirm their roles and to understand the underlying molecular mechanisms of their inhibitory effects. We used flow cytometry analysis to detect changes in cell cycle patterns in HepG2 cells that were treated with the LC-antisense species for 48 h. When compared to control treatments, using lipids alone or LCSS plus lipids, 53 (~95%) out of the 56 LC-antisense molecules exhibited an increased percentage of cells with sub-G0-G1 DNA content (Fig. 4a). We then determined whether cell death caused by antisense treatment reflected the induction of apoptosis. HepG2 cells treated with LC-antisense molecules were subjected to a DNA fragmentation assay. Twenty seven (LCAS 1, 3, 5, 8, 11, 12, 14, 15, 16, 20, 21, 22, 24, 29, 31, 33, 34, 35, 40, 41, 42, 43, 47, 48, 49, 51, and 53) out of the 53 LC-antisense species were found to cause characteristic DNA ladder formation 48 h after the transfection, indicating apoptotic progression caused by the antisense molecules (Fig. 4b). These results suggest that the LC-antisense library system is an effective means for *en masse* identification of genes involved in cancer cell growth.

Functional validation of the identified genes using other antisense

A large number of genes were rapidly identified to be involved in liver cancer cell growth with the LC-antisense library. Functional validations of the genes identified from the liver cancer cells were further carried out by using other antisense technologies including siRNA and PS end-capped AS-oligos. We chose an LC-antisense of clone WGSL 11 (accession number BC024270; gene description, *H. sapiens* cutaneous T-cell lymphoma-associated tumor antigen), as an example, out of the seven LC-antisense molecules that showed differential inhibition of HepG2 cell growth (data not shown).

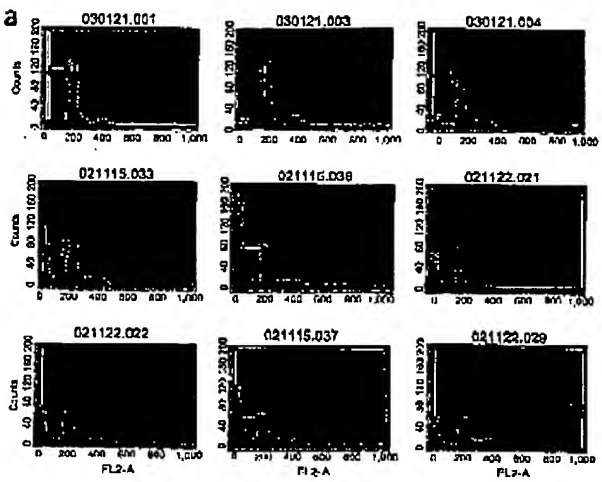

Figure 4 Effects of LC-antisense on cell cycle progression and apoptotic induction. A representative example of the data acquired from treatments with six kinds of LC-antisense is shown. (a) Cell cycle analysis after transfection of cells with LC-antisense molecules. HepG2 cells and controls were treated with the LC-antisense molecules, LCAS 11, LCAS 14, LCAS 15, LCAS 16, LCAS 29 and LCAS 31; sham treatment, lipids alone and control DNA + Li, (lipid) complexes. Cells were harvested at 48 h after transfection. Functional analysis was performed on an equal number of cells (10^4 events) by flow cytometry after staining of DNA with propidium iodide. (b) Induction of apoptotic DNA ladder formation by LC-antisense molecules. The LC-antisense molecules, LCAS 11, LCAS 14, LCAS 15, LCAS 16, LCAS 21 and LCAS 29, were treated to HepG2 cells along with controls: sham treatment, control DNA + Li. (lipid) complexes and cisplatin (positive control). Genomic DNA was extracted 48 h after transfection and run on a 1.6% agarose gel. M, 100 bp DNA ladder size marker.

We studied the effect of two siRNAs, 11-1siRNA and 11-2siRNA, to WGSL 11, on target mRNA level. 11-1siRNA, was found to be effective in target RNA reduction, and was compared with LCAS 11 for target RNA reduction and cell proliferation blockade. Quantitative downregulation of the target gene expression by LCAS-11 and 11-1siRNA was done with real-time RT-PCR to detect target mRNA levels in HepG2 cells. 24-h treatment with LC-antisense (1.6 nM) and siRNA (50 nM) resulted in about a 70% and 60% reduction of target mRNA, respectively, when compared to that obtained with sham treatment (Fig. 5a). HepG2 cell growth was inhibited by about 70% and 67% by 0.8 nM LCAS-11 and 25 nM 11-1siRNA, respectively (Fig. 5b).

Next, we set out to reconfirm the validity of the functional data by using other types of antisense molecules. To find an effective target site for antisense inhibition, a series of PS end-capped AS-oligos derived from the clone WGSL11 were designed and evaluated for their ability to inhibit target gene expression and HepG2 cell growth. Of five antisense sequences tested, the most active one, PS 11-1, was selected. When treated with PS 11-1, target mRNA levels were much reduced and cell growth was inhibited by about 70% at a concentration of 1.1 μM (Fig. 5c,d). In contrast, cells treated with either mismatch or sense control end-capped AS-oligos did not show much reduction of target mRNA and resulted in only marginal cell growth inhibition. The result was reconfirmed by Southern hybridization of the RT-PCR band of target mRNA (Fig. 5e). These results validate the utility of LC-antisense library in screening genes of interest in a high-throughput mode.

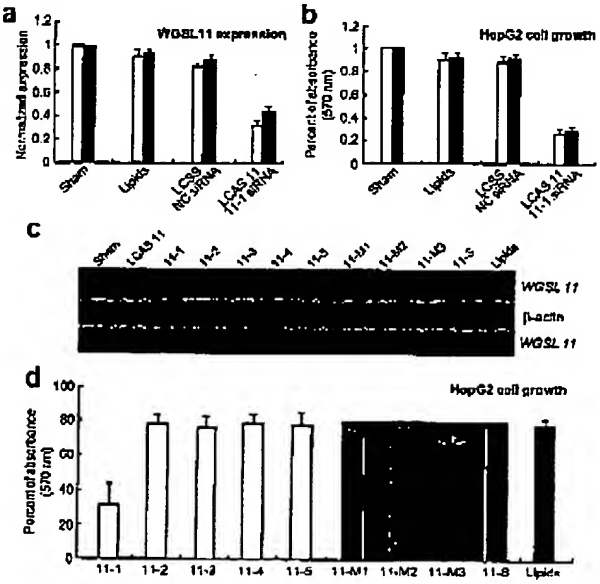

Figure 5 Validation of WGSL 11 as a potential target for the inhibition of cancer cell growth. LCAS 11, siRNA and a series of PS end-capped AS-oligos directed against WGSL 11 were designed and transfected into HepG2 cells. (a) HepG2 cells were treated with 1.6 nM LCAS 11 or 50 nM 11-1siRNA or controls in a 24-well plate. Total RNA was isolated and subjected to real-time RT-PCR 24 h after the antisense treatments. Values represent the average of three independent experiments. Open bars, treatment by LCAS 11 or controls; solid bars, treatment by 11-1siRNA or controls. (b) HepG2 cells were treated with 0.8 nM of LCAS 11, 25 nM of 11-1siRNA or controls in a 96-well plate. The transfectants were examined for growth inhibition using MTT assays 72 h after the antisense treatments. Cells treated with sham, lipids alone and control molecules complexed with lipids were also assayed for comparisons. Open bars, treatment by LCAS 11 or controls; solid bars, treatment by 11-1siRNA or controls. (c) HepG2 cells were transfected with 0.8 nM LCAS 11 and 1.1 μM the PS end-capped AS-oligos, complexed with Lipofectin in a 48-well plate. Total RNA was subjected to RT-PCR 48 h after the AS-oligo treatments. DNA bands in b were then transferred onto a nylon membrane and subjected to Southern hybridization. (d) HepG2 cells were transfected with each PS end-capped AS-oligos (0.16 μM) complexed with Lipofectin at a ratio of 1:2.5 (wt/wt) in a 96-well plate. MTT assays were carried out to determine the inhibition of cell growth 72 h after AS-oligos treatment. Each bar value represents the mean ± s.d. of triplicate experiments. Sham, sham treated; 11-1–5, treated with PS end-capped AS-oligos of five different sequences; 11-M1–M3, treated with mismatch control oligos of three different sequences to PS 11-1; and 11-S, treated with a sense control oligo of PS 11-1.

DISCUSSION

LC antisense molecules were generated as single-stranded genomic DNA of recombinant bacteriophages, and was tested for stability, antisense activity and, further, for its usefulness in high-throughput functional genomics. When LC-antisense molecules to *TNF-α* mRNA were used, the antisense molecules were found to be stable in the presence of nucleases and effective in reduction of target mRNA, which was reflected in the antisense activity that required less than 1/10 the amount of most other types of AS-oligos. LC-antisense to *TNF-α* was also found to substantially reduce production of rat TNF-α in cells, confirming commensurate antisense activity at the protein level. Further, the broad utility of LC-antisense was confirmed with antisense-mediated expression blockade of several other genes (*c-myc*, *c-myb*, *cdk2* and *cdk4*) of biological significance.

The enhanced antisense activity of LC-antisense molecules may be explained in two ways. One reason may be that the long antisense sequence (1,000 bases on average) in the molecules allows the formation of a more stable duplex between the antisense sequence and the complementary sequence of target mRNA. The lengthy duplex may serve as a substrate for RNaseH activity for an extended period of time. Another reason is that mRNA tends to form extensive secondary and tertiary structures among its own sequences or interact with RNA-binding proteins in the cell cytoplasm, which can make some target sequences inaccessible. It is more likely, with its long length, that certain regions within LC-antisense molecules have a higher chance of binding to complementary sequences in target mRNA.

As the antisense sequences are much longer in LC-antisense molecules, target-specificity of LC-antisense is of critical concern. To prove sequence specificity of LC-antisense in a rigorous manner, we carried out both multi-probe RPA and real time RT-PCR. The specific antisense activity was shown by the lack of off-target effects between LC-antisense molecules to *c-myb* and *c-myc* and was reconfirmed by LC-antisense molecules and siRNA targeting the same gene, *cdk2*. Because *cdk4* expression was downregulated only by *cdk4* LCAS but not by *cdk2* LCAS, and vice versa, also indicates sequence specificity because the two genes have a region of localized homology. The delayed downregulation by functionally associated genes may be explained by the tightly coordinated regulation between cell cycle regulatory proteins. Perturbed expression of a growth regulatory gene has been reported to alter expression of other genes involved in the G1/S transition phase of cell cycle progression[36]. Primary reduction of *cdk2* expression may have subsequently lowered the activity of CDK4 (ref. 35). Even with its long length, LC-antisense provides sequence specificity comparable or better to existing antisense technologies. As with other antisense technologies including siRNA, there may still be some off-target effects when a large amount of LC-antisense molecules is used.

Knockdown conferred by antisense provides much faster means for gene functionalization than do the conventional knockout methods. To take advantage of antisense technologies, antisense libraries have been constructed using AS-oligos and used for selection of drug targets[39,40]. A vector system for expression of ssDNA in mammalian cells was also reported[41]. More recently, an approach using a genome-wide synthetic siRNA or siRNA expression library was developed for unveiling gene functions[42]. Although the technology of siRNA has been reported to be effective, the efficiency in target reduction and specificity appears to be comparable to those of LC-antisense. It should be noted that both AS-oligos and siRNA, unlike LC-antisense, require target site searches that are time consuming and often inconclusive. LC-antisense, as with other antisense, appears to bring about somewhat varying degrees of target reduction even with its lower variability and better efficiency. Thus, antisense activities obtained from distinct LC-antisense molecules need to be analyzed with some prudence.

By using the random gene LC-antisense library, we identified 56 genes functionally involved in liver cancer cell growth. Motif-based searches suggested that these include genes with novel functions and genes with defined functions, some of which were, as expected, involved in critical cellular metabolisms in DNA replication, transcription and translation. Yet, some others contribute to cancer cell growth in addition to their functions that appear irrelevant to cancer cell growth. Several genes that we found to be involved in liver cancer cell growth in the present study were shown to be overexpressed in hepatocellular carcinoma[43,44]. In fact, ribosomal proteins P1, S17, L35, fibrinogen gamma polypeptide and elongation factor-1 gamma (WGSL 23, 38, 55, 40 and 41 respectively) were overexpressed in liver cancer and involved in protein synthesis. These results suggest that these genes, although essential in their housekeeping roles, may have differential expression levels in liver cancer tissues and support cell growth in liver cancer. Similar findings of a large number of housekeeping genes in the identification of growth-related genes were also reported in other functional genomics using expressed antisense in *Candida albicans*[45]. If overexpressed above a normal level, these genes may play an important role in cancer or pathogenic cell growth. Recently, for example, α-fetoprotein (WGSL 8) was reported to stimulate expression of some oncogenes (*c-fos*, *c-jun* and *N-ras*) in liver cancer cells[46]. It would then be worth targeting these genes to curb cancer cell growth. Further studies are clearly warranted to investigate biochemical processes of protein products of the genes.

An LC-antisense library can be constructed with the unidirectional cloning of cDNA fragments of known sequences (unigenes) into phagemid vectors. Contrary to the random gene antisense library, each antisense species in the unigene antisense library has a unique sequence. An advantage of the unigene antisense library is that a large panel of human genes can be individually targeted without redundancy, and genes of constant transcription levels with post-translational modifications may be screened.

The LC-antisense library system may provide a faster, more cost effective and analytically accurate tool for the study of functional genomics. It would be interesting to see if the LC-antisense molecules can also be used in animals as this would potentially provide a useful approach for *in vivo* functional genomics.

METHODS

Construction of recombinant phagemids. Various recombinant phagemids were constructed according to standard cloning procedure[47]. WRT7/P2 cells (1×10^5) were seeded in each well of a 48-well plate. Rat *TNF-α* expression was induced in the cells by the treatment of LPS (Sigma-Aldrich) at 30 μg/ml for 4–24 h. Cells were harvested at desired time points to examine the level of mRNA. The LPS incubation time that induced the highest expression level of TNF-α was chosen for further experiments. The RT-PCR fragment (708 bp) of *TNF-α* that comprises the entire coding sequence was amplified with a pair of PCR primers (5′-GATCGTCGACGATGAGCACAGAAAGCATGATCC-3′ and 5′-GATCGAATTCGTCACAGAGCAATGACTCCAAAG-3′) and sequence verified. To construct *TNFα*-LCAS, the rat *TNF-α* cDNA fragment was cloned into the multiple cloning site of the pBluescript (pBS) KS(−) vector (Stratagene) using *Sal*I and *Eco*RI restriction sites in the same direction as the *lacZ* gene (see Supplementary Fig. 1 online). Control sense molecules were constructed similarly. Likewise, cDNA fragments of the *c-myc*, *c-myb*, *cdk2* and *cdk4* genes were amplified with a pair of PCR primers (see Supplementary Table 1 online) and cloned into the *Eco*RV site of pBS-KS (+) or (−) vector. The recombinant phagemids were transformed into *Epicurian Coli* XL-1 Blue competent cells (Stratagene) by the calcium-chloride method. Cloning direction of amplified cDNA fragments were confirmed with both restriction digestion and DNA sequencing.

Production and purification of either LC-antisense or control molecules. LC-antisense or control molecules to target genes were produced by overnight culture of transformed bacterial cells that had previously been infected with helper bacteriophages, and purified by gel filtration column chromatography. These methods are described in detail in Supplementary Methods online.

Structural analysis and stability test of LC-antisense molecules. For structural analysis, 1 μg of *TNFα*-LCAS was treated with *Xho*I (10 U/μg DNA), exonuclease III (160 U/μg DNA), or S1 nuclease (10 U/μg DNA) at 37 °C for 3 h, and subjected to phenol extraction, ethanol precipitation and gel electrophoresis on a 1% agarose gel. For stability test, 1 μg of the antisense molecules was tested alone or after complex formation with lipids at a ratio of 1:3 (wt/wt) of DNA/lipids. We added 30% FBS that was not heat inactivated to the antisense-lipids complex and incubated it at 37 °C for varying periods of time for up to 48 h. After incubation with FBS and exonucleases, LC-antisense was extracted with phenol, precipitated with ethanol and run on a 1% agarose gel.

Transfection of LC-antisense and siRNA. Transfection of LC-antisense or siRNA was carried out to study the activity of the LC-antisense molecules. For LC-antisense transfection, cells were seeded on a 6-well (for RPA assay), 24-well (for real-time PCR) or 48-well plate (for RT-PCR) in an appropriate volume of culture medium. Cationic lipids, Lipofectamine, Lipofectamine 2000 or Lipofectamine plus reagents (Invitrogen) were mixed with the purified molecules in various ratios (wt/wt) for transfection into target cells. These lipid-DNA complexes were mixed with Opti-MEM (Invitrogen) and added to cells according to the manufacturer's protocol. After 6 h transfection at 37 °C, the cells were added with fresh medium and incubated further for up to 48 h at 37 °C before assays. Expression of the Rat *TNF-α* gene was induced with LPS treatment (30 μg/ml) to WRTP7/P2 cell transfectants. To compare the effects of the LC-antisense molecules, identical quantities of lipids alone and control DNA plus lipid complexes were also added to the same number of cells in a different well plate and assayed simultaneously. For siRNA transfection, cells were seeded on a 24-well (for real-time PCR), or 96-well plate (for MTT reduction assay) in an appropriate volume of culture medium. *Cdk2*siRNA (sense sequence, 5′-GGUACCGAGCUCC UGAAAUCTT-3′; antisense, 5′-GAU UUCAGGAGCUCGGUACCTT-3′), *11-1*siRNA (sense sequence, 5′-GCAGG CACUGGAGGAUAUUCTT-3′; antisense, 5′-GAAUAUCCUCCAGUGCCUG CTT-3′), and *11-2*siRNA (sense sequence, 5′-GAAGCAAGAAAUGAAGAAAC TT-3′; antisense, 5′-GUUUCUUCAUUUCUUGCUUCTT-3′) duplexes were synthesized (Bioneer) and transfected into HeLa or HepG2 cells using siPORT Lipid (Ambion) as recommended by the manufacturer. To compare the effects of the siRNA molecules, identical quantities of lipids alone and negative control no. 1 siRNA (Ambion) plus lipid complexes were also added to the same number of cells in a different well plate and assayed simultaneously.

Detection of target gene transcript. After the transfection of LC-antisense or siRNA, the change of target gene expression in mRNA level was detected with RT-PCR, RPA, and real-time quantitative RT-PCR methods. RNA preparation was carried out with Tri reagent (Molecular Research Center) according to the protocol recommended by the manufacturer. Purified RNA was subjected to RT-PCR in a 50-μl reaction volume by using the Access RT-PCR kit (Promega) and a thermal cycler (MJ Research) as recommended by the manufacturer. A pair of primers was used to amplify *TNF-α*, *c-myc*, *c-myb* and *WGSL 11* genes (see Supplementary Table 2 online). PCR product was confirmed on a 1% agarose gel, and quantitative analysis of the amplified DNA was performed with AlphaImager 1220, a gel documentation apparatus (Alpha Innotech).

To investigate the effect of LC-antisense molecule on the steady-state level of *cdk2* mRNA, RPA was carried out according to the instruction of the RiboQuant Multi-Probe RPA System (BD Pharmingen). Total RNA was obtained from the transfectants at 24 h and 48 h after antisense or control treatment. The antisense RNA probes were synthesized from an hCC-1 template set (BD Pharmingen) in the presence of biotin-16-UTP (Roche). The biotin-labeled probes were hybridized in excess to 20 μg total RNA in solution. Unprotected probes and RNA were digested by RNases. The RNA/probe hybrids were run on a denaturing polyacrylamide gel and then transferred onto a nylon membrane by a semi-dry blotting unit (Fisher Scientific). Immobilized hybrids were cross-linked to the membrane by exposing to UV light. The membrane was incubated with a streptavidin-horseradish peroxidase reagent before exposure to an X-ray film.

Target gene expression was also measured by real-time quantitative RT-PCR. Total RNA (1 μg) was reverse transcribed by using random primers supplied in the Reverse Transcription System (Promega). To quantify gene expression, cDNA of *cdk2*, *cdk4*, *cdk6* and *WGSL 11* genes were amplified by using respective pair of primers (see Supplementary Table 3 online), the DyNAmo HS SYBR Green qPCR Kit (MJ Research), and the DNA Engine Opticon 2 System (MJ Research) according to the manufacturer's instruction. To normalize the amount of total RNA present in each reaction, β-*actin* gene was amplified simultaneously. Triplicate assays were done with RNA samples isolated from at least two independent experiments.

Detection of polypeptides with ELISA or western blotting. Quantification of each target protein after *TNFα*-LCAS, *cdk2*-LCAS, and *cdk4*-LCAS treatment was performed with the enzyme-linked immunosorbent assay (ELISA) or western blotting analysis (see Supplementary Methods online).

MTT assay to determine inhibition of cell growth. Both LC-antisense molecules (*c-myb* and *cdk4* LCAS) and siRNA to WGSL11 were studied for their growth inhibitory effects using the MTT assay. The MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich) was diluted with PBS to a concentration of 5 mg/ml, and 100 μg of the diluent was added to each well containing 100 μl culture medium. Cells were maintained in a CO_2 incubator at 37 °C for 4 h and treated with an equal amount of isopropanol (containing 0.1N HCl) at 25 °C for 1 h. The cells were then measured for absorbance at 570 nm with an ELISA reader, SpectraMAX 190 (Molecular Devices).

Construction of a unidirectional subtracted liver cDNA library. To clone differentially expressed genes in hepatoblastomas, a cDNA library was constructed by using a subtractive hybridization procedure[17]. The construction of the library is described in detail in Supplementary Methods online.

Preparation of a liver cancer LC-antisense library. Bacterial competent cells containing recombinant pBS SK(−) phagemids were plated on Luria-Bertani agar plates containing 50 μg/ml of ampicillin and 50 μg/ml of tetracycline and incubated at 37 °C for 16 h. Isolated colonies were seeded in a well of 96-deep-well plates containing 1.4 ml of 2×YT liquid medium (tryptone 16 g, yeast extract 10 g, NaCl 10 g per 1,000 ml) added with 50 μg/ml ampicillin. Cells were cultured for 7 h at 37 °C with vigorous shaking. To produce LC-antisense molecules from each phagemid, 20 μl of the bacterial culture was transferred to each well prefilled with 1.4 ml of fresh 2×YT liquid medium containing 9 μl of helper bacteriophages, M13K07 (New England Biolabs). After 1 h incubation, 4.2 μl of 70 μg/ml kanamycin was added and cultured at 37 °C for 12 h. The superinfection was carried out in triplicate for each clone to maximize the yield of antisense molecules in a single purification step. Single-stranded LC-antisense molecules were purified from the culture supernatant of bacterial cells using QIAprep 96 M13 Kits and QIAVAC vacuum manifolds (Qiagen) according to manufacturer's instructions. To test both quantity and purity, we ran purified LC-antisense molecules on a 1% agarose gel along with control LC-molecules derived from pBS SK(−) phagemid without a cDNA insert.

Transfection of an LC-antisense library into a liver cancer cell line. To identify genes involved in the growth of liver cancer cells, Lipofectamine 2000 was mixed with antisense molecules of the liver cancer–specific LC-antisense library for transfection into HepG2. The cells (7×10^5) were washed twice with Opti-MEM, seeded in each well of 96-well plates in 100 μl of Opti-MEM supplemented with 10% FBS and incubated for 12–18 h at 37 °C in a 5% CO_2 incubator. LC-antisense molecules (0.1 μg) were complexed with 0.3 μg of the cationic lipids, and the antisense molecule plus lipid complexes were added to the cultured cells. The cultures were exchanged with fresh medium 24 h after transfection and incubated for 4 d further. To compare the effects of the LC-antisense molecules on cell proliferation, we also added equal quantities of lipids alone and control DNA plus lipid complexes to the same number of cells in a different 96-well plate and assayed them simultaneously. Control DNA was single-stranded phage genomic DNA lacking a cDNA insert. After the transfection, microscopic observation or MTT reduction assay was performed to study

the effect of antisense molecules on proliferation of cancer cells as described above. The percentage of growth inhibition of cells in each well treated with antisense plus lipids complex was calculated by comparing the optical density with those of sham treatments, using the following formula: 1 − (absorbance of an experimental well/absorbance of a sham control well) × 100.

Gene identification and sequence motif search. To identify genes complementary to LC-antisense molecules that inhibited proliferation of liver cancer cells, we sequenced recombinant phagemids obtained by alkaline lysis from the 5′ upstream of the (+) strand of cDNA inserts using the T3 primer. Sequences of cDNA inserts were compared with those of the GenBank database. Polypeptides deduced from cDNA sequences were then searched for amino acid motifs using the ProfileScan Server (http://bits.isb-sib.ch/cgi-bin/PFSCAN).

Treatment of PS end-capped AS-oligos. To reconfirm the functional role of *WGSL11* gene in the cell proliferation of liver cancer, we designed a series of PS end-capped AS-oligos (see **Supplementary Table 4** online) and transfected HepG2 cells. The procedures are described in detail in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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